Cytometry Part B (Clinical Cytometry) 78B (Suppl. 1):S47–S60 (2010)

Review Article

Human Peripheral Blood B-Cell Compartments: A Crossroad in B-Cell Traffic

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A relatively high number of different subsets of B-cells are generated through the differentiation of early B-cell precursors into mature B-lymphocytes in the bone marrow (BM) and antigen-triggered maturation of germinal center B-cells into memory B-lymphocytes and plasmablasts in lymphoid tissues. These B-cell subpopulations, which are produced in the BM and lymphoid tissues, recirculate through peripheral blood (PB), into different tissues including mucosa and the BM, where long-living plasma cells produce antibodies. These circulating PB B-cells can be classified according to their maturation stage into i) immature/transitional, ii) naïve, and iii) memory B-lymphocytes, and iv) plasmablasts/plasma cells. Additionally, unique subsets of memory B-lymphocytes and plasmablasts/plasma cells can be identified based on their differential expression of unique Ig-heavy chain isotypes (e.g.: IgM, IgD, IgG, IgA). In the present paper, we review recent data reported in the literature about the distribution, immunophenotypic and functional characteristics of these cell subpopulations, as well as their distribution in PB according to age and seasonal changes. Additional information is also provided in this regard based on the study of a population-based cohort of 600 healthy adults aged from 20 to 80 years, recruited in the Salamanca area in western Spain. Detailed knowledge of the distribution and traffic of B-cell subsets through PB mirrors the immune status of an individual subject and it may also contribute to a better understanding of B-cell disorders related to B-cell biology and homeostasis, such as monoclonal B-cell lymphocytosis (MBL). © 2010 International Clinical Cytometry Society

Key terms: B-cell; peripheral blood; circulating; differentiation; subsets

Additional Supporting Information may be found in the online version of this article.

Grant sponsor: MSCNet European strep; Grant number: N° E06005FF; Grant sponsor: Fondo de Investigación Sanitaria, Instituto de Salud Carlos III, Ministerio de Sanidad y Consumo, Madrid, Spain; Grant numbers: RTICC RD06/0020/0035, PI06/0824, PS09/02430; Grant sponsor: Gerencia Regional de Salud de castilla y León; Grant number: GRS206/A/08; Grant sponsor: Ayuda GR37 de Excelencia de Castilla y León, Consejeria de Educación, Junta de Castilla y León, Valladolid, Spain; Fundación Samuél Solorzano, Salamanca, Spain; Grant number: FS/16-2008.

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Received 17 March 2010; Revision 17 May 2010; Accepted 1 June 2010

Published online in Wiley Online Library (wileyonlinelibrary.com).
DOI: 10.1002/cyto.b.20547

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How to cite this article: Perez-Andres M, Paiva B, Nieto WG, Caraux A, Schmitz A, Almeida J, Vogt RF, Marti GE, Rawstron AC, Van Zelm MC, Van Dongen JJM, Johnsen HE, Klein B, Orfao A. Human peripheral blood B-cell compartments: A crossroad in B-cell traffic. Cytometry Part B 2010; 78B (Suppl. 1): S47–S60.

B-cell development takes place in two stages which are localized in different tissues: (i) the differentiation of B-cell precursors from an hematopoietic stem cell (HSC) to naïve mature B-lymphocytes in the bone marrow (BM) (1), and (ii) the maturation to memory/effector cells in secondary lymphoid tissues–lymph nodes (LN) and mucosa-associated lymphoid tissues (MALT)-, the BM and spleen (2,3). Traffic of the resulting B-cell subsets between these tissues through peripheral blood (PB) mirrors the immune status of an individual subject and potentially also disorders of B-cell development, autoimmunity, and lymphoproliferative diseases related to B-cell biology and homeostasis.

Competition among B-cell precursors in the BM for limited life-span and differentiation-promoting resources (e.g., lineage-specific cytokines, stromal cell surface receptors, and B-cell antigen receptor [BCR] ligands) leads these cells through stepwise differentiation from HSCs to functionally mature B-lymphocytes (1,4). Naïve B-cells with mature phenotypic characteristics and a fully functional BCR (5) leave the BM through blood vessels, reach PB, and recirculate between the secondary lymphoid tissues, dying after a few days. Those naïve B-cells recognizing an antigen (Ag) in the LN, spleen, or MALT will be primed and start the germinal center (GC) reaction in collaboration with activated CD4+ T-cells (2,3). After following several rounds of proliferation and affinity maturation in the GC dark zone and the GC light zone, these cells generate memory and pre-effector (plasmablasts) B-cells (2,3). Quiescent memory B-cells remain recirculating in the PB or they migrate to Ag-draining tissues (6). Meanwhile, the plasmablasts look for survival niches in the BM or the MALT to complete their differentiation to antibody (Ab)-secreting cells—plasma cells (PC) (7).

For many years, full clear phenotypic profiles of the different stages of B-cell maturation in the BM (5) and the LN (2) have been defined in humans which form the basis of the classification of B-cell leukemias/lymphomas originating in these tissues. In contrast, because of their phenotypic complexity, PB circulating B-cell subsets have been poorly defined until recently. Classification of these cells has been traditionally based on murine models (8) that follow a slightly different biology with at least five major general differences: (i) the adult mouse BM is significantly more hypercellular; (ii) the mouse spleen is normally a site of some extramedullary hematopoiesis; (iii) chronic lymphoid leukemias are not prominent in mice; (iv) the classification of mouse lymphomas is based on the spleen and not the lymph node, and (v) mice are kept under specific pathogenic free conditions and have mostly naïve B-cell compartment. As an example of such differences, identification of CD27 as a marker restricted in human PB samples to cells that have experienced antigen (9,10) has allowed the identification of circulating memory B-cells and plasmablasts in humans and it has been of great utility for the analysis of human B-cell biology in steady-state blood, after immunological priming (infection/vaccination) and in different B-cell disorders (autoimmunity/neoplasia). In contrast, CD27 is not expressed by the murine counterpart of these human B-cells (11). In turn recent studies have described that the circulating pre-GC B-cells comprise not only matured naïve B-lymphocytes but also a minor subpopulation of B-cells with lower functional abilities and immature phenotypic features (12–14). Although these cells were firstly identified as the human phenotypic counterpart of murine transitional B cells (12), latter reports showed that these cells are heterogeneous and have intermediate functional/phenotypic characteristics between those of maturing BM precursors and naïve B-lymphocytes (15). These observations support the notion that these pre-GC B-cells leave the BM prior to completing the negative selection process. This could presumably be due to the inability of BM to retain all B-cell precursors at the latest maturation stages, as more clearly observed in patients under BM regeneration (16,17). Consistent with this hypothesis, an increased frequency of immature B cells is also observed in the PB of patients with autoimmune diseases (12–14).

In this article, we review recent data reported in the literature about PB B-cells and expand this information with additional data derived from the study of 614 healthy adults aged from 20 to 90 years (a population-based cohort recruited in the Salamanca area in western Spain; see Supporting Information).

B-CELL DIFFERENTIATION AND MATURATION IN THE BM

BM stromal cells are key elements in the development of B-cells providing specific cell–cell contacts and soluble cytokines (1,18). These stromal cells build a network in the intersinusoidal space of the bone cavity that extends from the endostium to the endothelial cell basement membrane of the sinusoids (1,19). These cells, together with the osteoblasts and osteoclasts (1,20), T-cells (21) and other leukocytes (i.e., dendritic cells) (22,23), form specific niches which provide those microenvironmental conditions required for each specific developing B-cell subset.

Based on the status of the immunoglobulin (Ig) chain genes and the expression of a variety of cell surface and

intracellular proteins, five major B-cell maturation stages have been identified among BM B-cell precursors: pro-B, pre-B-I, pre-B-II, immature, and naïve B-cells (1,4,5,18,24). During the first steps of B-cell differentiation, BM precursors are committed into the B-cell linage through VLA-4/VCAM-1 and c-kit/SCF interactions with stromal cells (1,4,25). These pro-B cells express CD22 and dim CD45 together with precursor markers such as CD34 and high levels of CD38; meanwhile, CD19 is still absent at this stage (5,24,26). Pro-B cells, attached to CXCL12-expressing cells through CXCR4, are not fully committed B-lineage precursors until they migrate to a different niche containing IL-7-expressing cells (1,20,27).

Interaction of IL-7 with its receptor (CD127) expressed on the B-cell progenitors promotes production of several transcriptional factors (e.g., Pax-5, PU.1, EBF-1, E2A). These transcriptional factors induce the synthesis of terminal-deoxynucleotidil-transferase (TdT) and the recombinase activating gene (RAG) products rag1 and rag2, which are required to start somatic recombination between the D and J gene segments of the Ig heavy (IGH) chain locus (28,29). Recombinase proteins (including rag-1 and rag-2) randomly select and assemble one segment from each of these two segments of genes deleting the DNA between them. Meanwhile, TdT randomly adds nucleotides to the joining exons, increasing the variability of the BCR (30). Upon expression of pax-5, the pro-B cells becomes committed to the B-cell lineage as pre B-I cells, where CD19 is firstly detected (CD19 is a direct target of pax-5) together with a $CD10^{hi}CD38^{hi}CD34^{+}CD79a^{+}$ phenotype and nuclear (n) TdT staining. During this step rearrangement of the IGH locus is completed with the joining of one V segment to the preformed D-J joint (30).

Upon generation of a functional VDJ exon, it will be spliced to the constant Igμ exons, generating an Igμ heavy chain. Those cells with detectable intracellular Igμ (cy-Igμ) are identified as pre B-II cells (5,31); they show heterogeneous surface staining for CD20, upregulation of CD45 expression and negativity for CD34 and nTdT (5,24). This Igμ will be expressed at low levels on the surface of pre-B cells together with the invariate surrogate light chain proteins VpreB and λ5. These two latter replace substitute for the Ig light (IgL) chain whose loci have not been rearranged yet at these early stages of B-cell development (18,32). The Igα and Igβ (CD79a and CD79b) signaling proteins are also expressed to form together with the Igμ chain, VpreB and λ5 the pre-B cell receptor (preBCR). If the preBCR is able to bind to the BM microenvironment, the ITAM (immunoreceptor tyrosine activate motifs) domains of Igα and Igβ are phosphorylated and their signaling rescues these cells from apoptosis (18,32,33). This positive selection confirms that the Igμ chain generated is functional, deleting those cells with unproductive IgH gene rearrangements (estimated to be around half of all generated pre-B cells) (18,30). Additionally, signaling through the pre-BCR induces proliferation of these B-cell precursors and rearrangement of the IGL genes (34).

Expression of a complete IgM molecule on the cell surface after both the IGH and IGL loci have been rearranged, is first detected in the immature B-cell compartment (5). These immature B-cells already share most of the phenotypic characteristics of naïve mature B-cells, including uniformly high expression of both CD20 and CD45, but unlike naïve mature B-cells they retain dim expression of CD10 and strong reactivity for CD38 (5). These cells pass through a negative selection process before they complete their maturation, to delete those cells with an autoreactive BCR (30,35,36). Immature B cells binding self antigens in the BM have the opportunity to rearrange their IGL loci again in order to edit their BCR (receptor editing) (30,35,36). If they are not able to produce a non-autoreactive BCR they will be deleted or become anergic depending on the affinity for self-antigens (18,30). Immature B-cells that pass successfully through this selection will become naïve mature B-cells with upregulation of the BCR negative-regulator CD22 molecule. In addition, these immature B-cells lose their CD10 and CD38 expression (5). Mature naïve B-lymphocytes leave the BM to PB and circulate through blood vessels.

In addition to the B-cell precursors (pro-B cells and pre-B cells), immature and naïve B-lymphocytes, plasma cells (PC) also reside in specific BM niches that support survival and long-term Ab-production (27,37). CXCL12 recruits CXCR4-expressing PC to the BM, where they receive stimulation through surface and soluble molecules produced by stromal cells and osteoclasts (26,37). The limited number of BM niches available means that newly generated plasmablasts have to compete for them with already settled PC (37)—and to a certain extent also with pro-B cells (27)—to become long-living PC.

Of note, although murine and human BM B-cell differentiation shares mechanisms and transcriptional factors, significant differences exist between the two (18). Most importantly, B-cell development in the murine BM leads to the production of an IgM+IgD- transitional stage that requires homing into the spleen to finish maturation to naïve B-cells (38), presumably because of the ongoing extramedullary hematopoiesis that may provide a BM like microenvironment in the mouse. In contrast, mature naïve B-cells that coexpress IgM+IgD+ and are fully functional are produced in human BM, and they do not depend on the spleen to complete maturation (5). In line with this hypothesis, normal number of circulating naïve B-cells has been reported in asplenic individuals who underwent surgery (39). However, this remains controversial since a slight increase in the absolute lymphocyte count after splenectomy potentially associated with a slight polyclonal B-cell lymphocytosis has also been reported by others (40–42)

ANTIGEN-DRIVEN B-CELL RESPONSES

The $CD27^{-}CD20^{+}CD19^{+}CD38^{-}$ naïve B-cells that exit the BM circulate through PB and enter the LN at the T-cell zone through high endothelial venules (2,3). If they do not encounter an antigen (Ag), they will leave the

Table 1
Distribution of Circulating B-Cells in Steady-State Peripheral Blood Samples from Healthy Adults (n = 614), According to their Maturation Stage

	Distribution of B-cells in adult PB									
	Mean		p10		p25		p75		p90	
	%	cells/μL	%	cells/μL	%	cells/μL	%	cells/μL	%	cells/μL
Total B-cells	2.5	157		63		89		203		272
Immature[a]	5.4	8.8	1.3	1.2	2.8	2.9	7.2	11.1	10	19.1
Naïve[b]	64	101	49	47	55	61	73	130	81	163
Memory[c]	31	52	14	16	21	26	40	65	44	96
Plasmablasts/Plasmacells[d]	2.1	3.0	0.4	0.6	0.8	1.0	2.3	3.1	4.4	5.9

Results are expressed as percentage of leukocytes or cells of the total B-cell in PB compartment, and absolute counts.
[a]$sIgM^{++}/CD10^{+d}/CD19^{+}/CD20^{+}/CD27^{-}/CD38^{++}/CD45^{+}$.
[b]$sIgM^{+}/CD10^{-}/CD19^{+}/CD20^{+}/CD27^{-}/CD38^{-/+}/CD45^{+}$.
[c]$sIgM^{-/+}/CD10^{-}/CD19^{+}/CD20^{+}/CD27^{+}/CD38^{-/+}/CD45^{+}$.
[d]$sIgM^{/+d}/CD10^{-}/CD19^{+}/CD20^{-/+d}/ CD27^{++}/CD38^{+++}/CD45^{+d}$.

LN through lymphatic vessels, recirculate between the PB and the lymphoid tissues, and they will die within several days. However, if the naïve B-cells encounter their cognate Ag presented by follicular dendritic cells (FDC) and they contact with activated Ag-specific T-cells, they become activated and migrate to a GC (2,3). In the GC, B-cells are continuously migrating between the dark zone (centroblasts) and the light zone (centrocytes). In the dark zone, cells undergo rapid proliferation, somatic hypermutation (SHM) of the IGV regions and IgH isotype class switch recombination (CSR); in the light zone, they re-encounter with the antigen to check that the former events have lead to an increased BCR affinity for the Ag (affinity maturation). This process results in the survival and proliferation of B-cell clones with a high-affinity BCR for the Ag (2,3). Upregulation of CD10, CD38, CD95, and HLA-DR and decreased CD44 and bcl2 expression discriminate GC B-cells from other B-cell subsets in LN (i.e., naïve and memory B-cells and plasmablasts) (2,43–45), together with a heterogeneous positivity for those markers related with maturation to the effector/memory compartment, such as CD27 (2,43,44). Although it has been suggested that centroblasts show higher CXCR4 (39) and CD77 (44) expression as compared with centrocytes, no clear consensus exists about the phenotypic differences between these two subsets (2). Afterward, B-cells committed to plasma cells upregulated the blimp, xbp-1 and irf-4 transcription factors, which repress the pax-5, bcl-6, mitf transcription factors governing the gene expression program that defines the naïve and GC B-cells. This new program inhibits future SHM and CSR, stimulates antibody secretion and changes in cell surface proteins and B-cell homing properties (2,3,46).

CIRCULATING PERIPHERAL BLOOD B-CELL SUBSETS: NUMERICAL DISTRIBUTION AND ORIGIN

Human naïve B-cells comprise about 60–70% of circulating B-cells (Table 1), have an unmutated IgV region sequence and they simultaneously co-express IgM and IgD (9–10). In contrast, memory B-cells (≈20–30% of all PB B-cells) have undergone SHM; they display mutations within their IgV regions, and about half have also undergone Ig CSR (9,10), as reflected by the expression of a switched sIgH (23% ± 10% and 21% ± 9% of adult PB memory B-cells express sIgG and sIgA, respectively,). The other half memory B-cells still coexpress sIgM and sIgD (52% ± 15% of memory B-cells), or potentially sIgM only. The origin of sIgM+sIgD+ memory B-cells remains a matter of debate. These memory B-cells were identified as a prediversified maginal zone population (47) that can be generated independently of functional GC (48). Furthermore, these cells have undergone less proliferation (six cell cycles) than GC cells (eight cell cycles) (49) with a lower number of somatic mutations (9,49). More recent studies, however, have shown that this population contains cells with the same phenotypic characteristics (50,51), and the same IgH gene rearrangements as found in Ig class-switched memory-B-cells of the same individual (52). Therefore, this population is likely to be a mixture of GC and GC-independent memory B-cells. sIgG+ and sIgA+ memory-B cells lacking CD27 have also been detected in blood of healthy individuals (53). Their origin and relation to the CD27+ memory-B-cell subsets are currently unknown, although the frequency of somatic mutations is lower (53). In line with these findings, we have observed that 21% ± 10% of sIgG+ and 9% ± 6% of sIgA+ memory B-cells from healthy adult PB do not express CD27. Up until now, no $sIgE^{+}$ memory B-cells have been found, and murine studies suggest that IgE-secreting PC could be generated directly from IgG_1 memory B-cells (54), although this possibility remains controversial (55).

During differentiation of naïve to memory B-cells, B-lymphocytes acquire a higher antigen binding affinity (3), at the same time they change the expression patterns of multiple surface receptors and intracellular factors that increase their responsiveness (51). Accordingly, memory B-cells show a higher in vitro response (*vs.* naïve B-cells) against different stimuli that mimic Ag-recognition (i.e., anti-BCR antibodies) and/or interaction with T-helper cells (i.e., CD40L) (51,56), with or without the support of cytokines (57) and/or TLR ligands (58). Once exposed to these factors, memory B-cells rapidly enter

the cell-cycle, they undergo more rounds of division and a larger proportion of them become Ab-secreting cells (plasmablasts and PC) (3,51,56-59). A publicly available B cell to plasma cell atlas (http://amazonia.transcriptome.eu/index.php?zone=PlasmaCell) has been recently provided, making it possible to quickly visualize the expression of a given gene throughout the differentiation of B-lymphocytes to plasma cells (60). Such differences in proliferation and maturation of naïve and memory B-cells in vitro mimic those of primary and secondary immune response in vivo. Of note, although both naïve and memory B-lymphocytes circulate in PB, many of the memory B-cells reside in places of antigen drainage such as the splenic marginal zone and the mucosa epithelium of the tonsils, while most of the naïve B-cells remain recirculating between the different lymphoid tissues (6,10).

Cells with morphological, phenotypical, and functional characteristics of PC are also detected at very low numbers in steady state adult PB (15,61,62). Most of these cells are believed to correspond to newly generated plasmablasts migrating from the secondary lymphoid tissues to a niche in the BM, MALT, or chronically inflamated tissues. Together with these circulating plasmablasts, mature PC that have left the BM and mucosa could presumably be detected inside this PB compartment under conditions of immune activation (61,62). This compartment represents about 1–3% (1–5 cells/μL) of all circulating PB B-cells in healthy adults in steady state conditions (15,61,62), although under specific conditions it can reach higher frequencies of all circulating B-cells (i.e. acute infections). In contrast to memory B-cells, IgA is the most frequently expressed surface immunoglobulin (sIg) on PB plasmablasts/PC (49% ± 12% of all PB plasmablasts/PC), with around 18% ± 12% of sIgM-only cells and 13% ± 11% $sIgG^+$ cells (15,61). About 14% ± 12% of all circulating PB plasmablasts/PC do not express any sIg (15,61). More intriguing is the presence of circulating sIgD+sIgM- plasmablasts/PC (<5% of plasmablasts/PC) in the PB of healthy adults (15); such sIgD+sIgM-plasmablasts/PC have been associated with immune responses in the upper respiratory tract (63,64). Notably, the Ig light chain distribution is also heterogeneous among the different compartments of circulating PB B-cells, plasmablasts/PC showing a lower κ/λ ratio *vs.* other B-cells (Fig. 1). Of note, sIgD+ only (sIgM negative) are often sIgλ expressing but are oligoclonal by DNA sequencing (65).

Although antigen-specific antibodies can be detected several decades after vaccination or infection, the half life of antibodies is much shorter, ranging from few days (i.e., IgA and IgM) to several weeks (i.e., IgG) (66). Humoral memory relies on continuous secretion of antibodies by long-living PC settled in survival niches (7,37). These niches will provide circulating PB PC with those factors required for their survival and further differentiation into long-living, mature PC (7,37). A competition between newborn plasmablasts and long-living PC that already reside in these niches causes most circulating

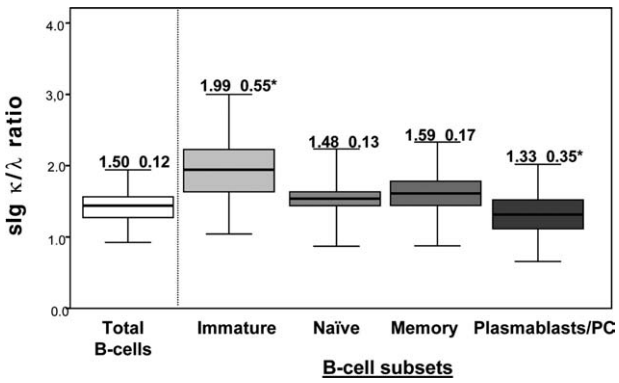

FIG. 1. Igκ/λ light chain ratio of the BCR expressed by B-cell subsets in peripheral blood of healthy adults. Numbers express mean ± one standard deviation. PC: plasma cells. *$P < 0.001$ vs. total B-cells.

plasmablasts to die; meanwhile, the replaced PC will recirculate through PB searching for homing in other niches (7). Accordingly, it has been recently shown that one week after tetanus toxoid vaccination mature PC can be detected inside this compartment of circulating PC, which have been replaced in the BM niches by new born plasmablasts generated in response to the tetanus vaccine (61,62).

As also previously reported (15,61,62), we have analyzed steady state PB samples from 614 healthy adults, who systematically displayed circulating plasmablasts/PC in the absence of apparent immune activation. Since most of these plasmablasts/PC express sIgA, it has been suggested that they are plasmablasts generated in response to mucosal commensal bacterial products and because of bacterial translocation at the gut (61,63,67). Antigens from the gut flora are a continuous source of activation for B-lymphocytes and other immune cells located in the Peyer Patches and the intestinal lamina propria, making this tissue not only the largest lymphoid organ, but also the major Ab-producing tissue (3–5 g/day) (63); in addition, these antigens can be filtered to local lymph nodes (63). B-cell stimulation sites at MALT and Ig-secretion PC niches are physically separated. Because of this, newly generated plasmablasts have to travel through the PB to their final location (63). Additionally, B-cells can be activated locally in the lamina propria by specific antigens independent of T-cell help and differentiate into IgA-secreting PC (68). Another mechanism that could explain the systematic observation of circulating plasmablasts/PC in every healthy adult subject is that these cells could be generated in the absence of antigen by polyclonal stimulation of memory B-cells through cytokines secreted by T-cells (69). In line with this latter hypothesis, some groups have reported a low, but significant, correlation between the number of circulating memory B-cells and the concentration of serum Ab (15,70), although this observation could not be confirmed in other studies (71). The fact that in rituximab-treated patients the decrease in the number of PB memory B-cells has no impact in the concentration of serum Ab (72), does not support the role of polyclonal

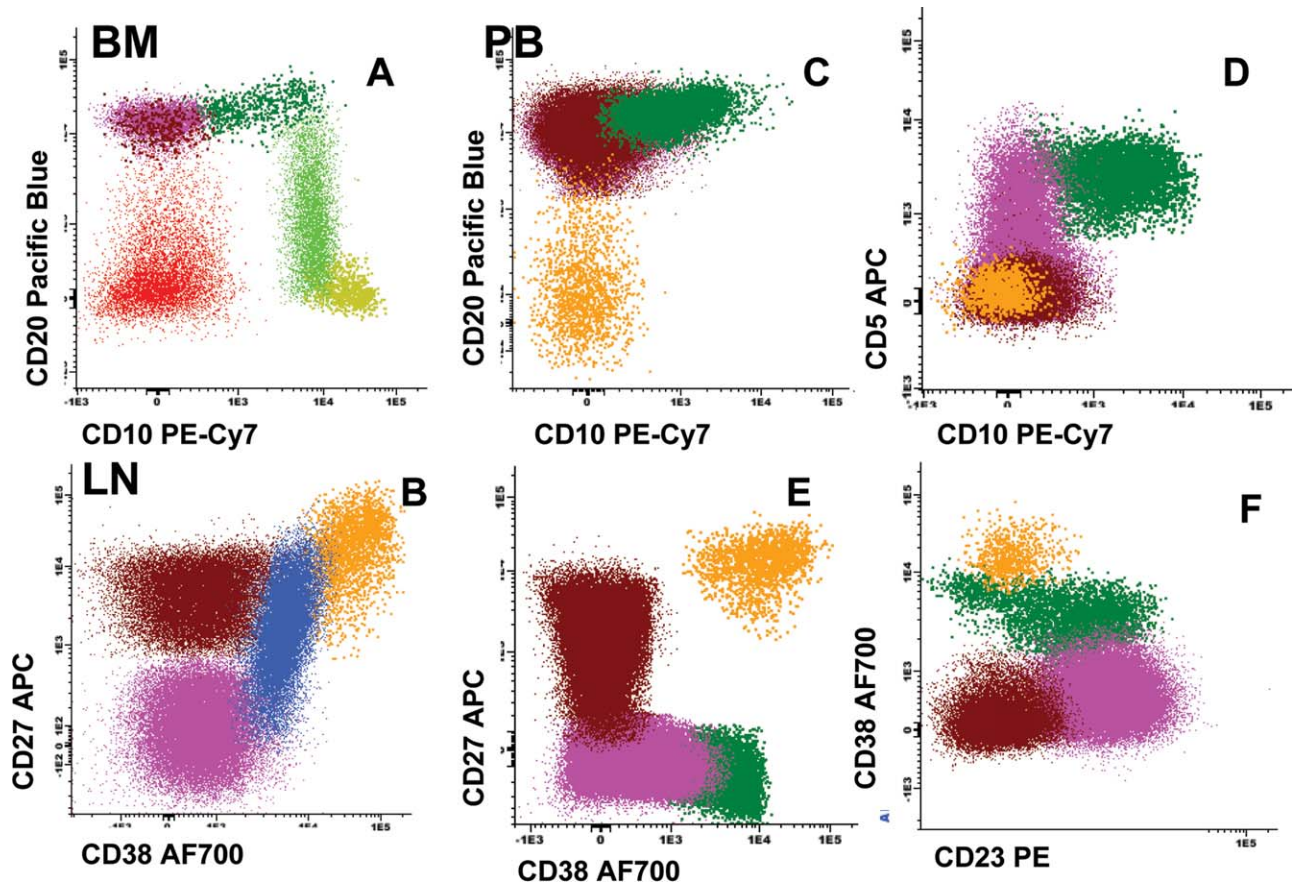

FIG. 2. Representative dot plots of normal maturation-associated phenotypes of B-cell subsets in human bone marrow (BM) (panel **A**), lymph nodes (LN) (panel **B**) and peripheral blood (PB) samples (panels **C–F**). B-cells and plasma cells were gated from cells showing $CD19^{+}$ and/or $CD38^{hi}$ expression and low-to-intermediate sideward light scatter (SSC) values. Color codes in the dot plots are as follows: light green, preB-I B-cell precursors in panel A; medium green, preB-II in panel A; dark green, immature B-cells in panel A and C to F; pink, naïve mature B-lymphocytes in panels A to F; blue, germinal center B-cells in panel B; maroon, memory B-lymphocytes in panels A to F; and orange, PB plasmablasts/PC in panels B to F; and red BM plasma cells in panel A. Note that in panel C the pink most naïve mature B-cells are hidden under memory B-cells.

stimulation of B-cells in the generation of long-living plasmablasts. Thus, the precise impact of these mechanisms (mucosal vs. polyclonal stimulation) in the daily generation of plasmablasts/PC, remains to be elucidated. In this regard, the pathway leading to production of plasmablasts/PC may be particularly important in both normal and pathological conditions, since in rheumatoid arthritis patients the pre and posttreatment levels of plasmablasts, but not of naïve or memory B-cells, closely correlate with the response to rituximab treatment, despite plasmablasts are not directly targeted by the antibody (73).

A minor subset of circulating immature B-cells has been also documented in normal human PB (12–15). Although these B-cells were first identified as the phenotypic counterpart of murine "transitional" B-cells, their characteristics and the differences in human vs. murine B-cell biology make it more likely that these cells correspond to immature B-cells leaving the BM during the negative selection process. In relative numbers, these immature B-cells account for 2–4% of all B-cells (1–5 cells per microliter of PB) in adults (12–14), they have unmutated IGH genes, express phenotypic features of immature B-cells (although most of them co-express sIgM and sIgD), and show a lower ability to proliferate and differentiate to Ab-secreting cells after in vitro stimulation, as compared with naïve mature B-cells (12–14). Of note, the κ/λ ratio is higher among these immature B-cells as compared with other PB B-cell subsets (Fig. 1). Although ex vivo survival of these cells can be improved reproducing the microenvironment of BM stroma support (e.g., when cultured with IL-4 and stromal cells), survival factors for naïve mature B-lymphocytes such as BAFF or TNFα do not show any significant effect (12–13).

A detailed analysis of this compartment of immune B-cells suggests that it is very heterogeneous, and includes cells leaving the BM at different steps between the immature and naïve mature B-cell stages with heterogeneous characteristics (74) (Fig 2). Of note, the frequency of these immature B-lymphocytes in PB has been found to be increased in autoimmune diseases and other immunological diseases (e.g., systemic lupus erythematosus, common variable immunodeficiency, X-

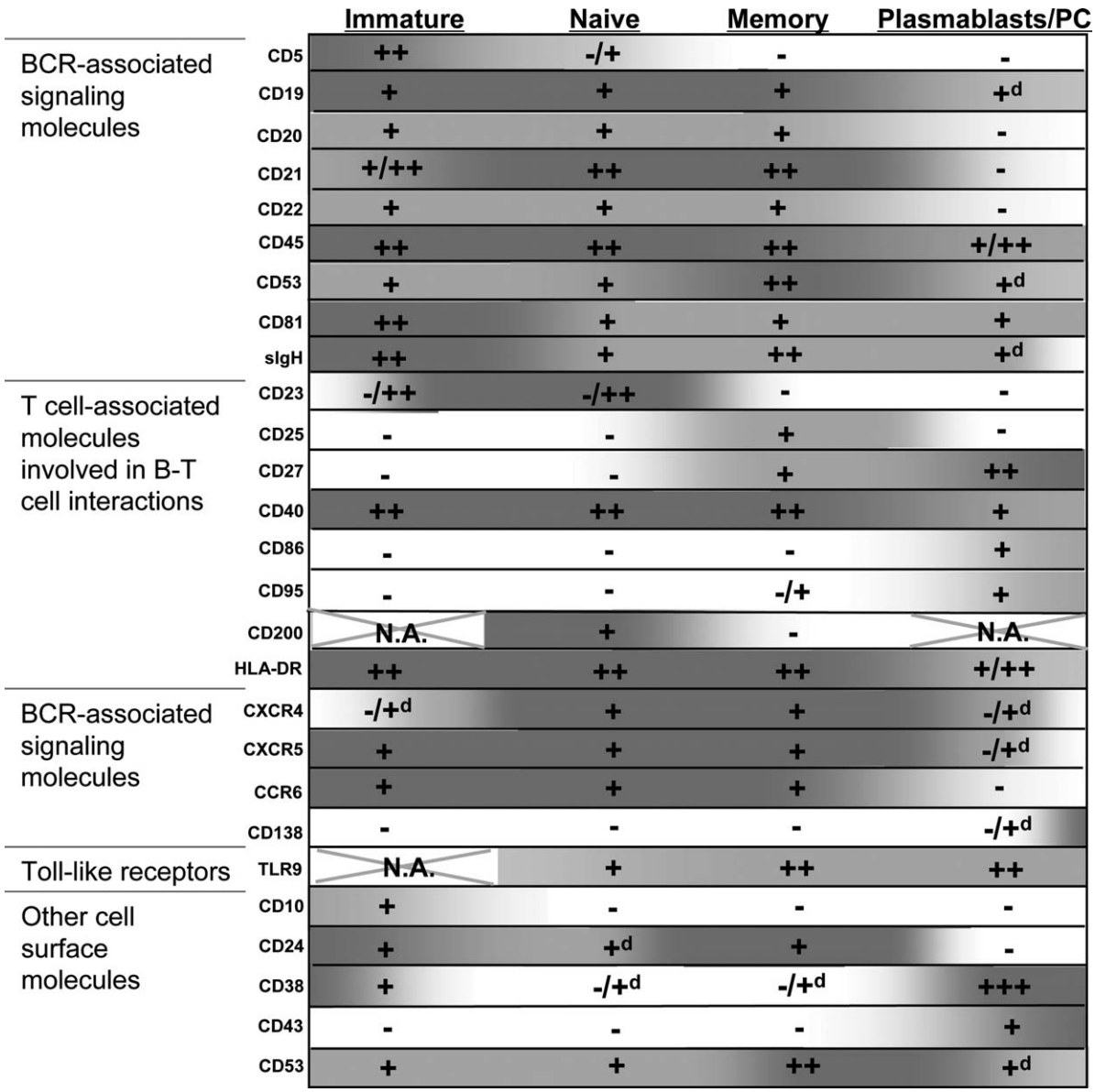
Immature
Naive
Memory
Plasmablasts/PC
BCR-associated signaling molecules
T cell-associated molecules involved in B-T cell interactions
BCR-associated signaling molecules
Toll-like receptors
Other cell surface molecules
CD5 ++ -/+ - -
CD19 + + + +d
CD20 + + + -
CD21 +/++ ++ ++ -
CD22 + + + -
CD45 ++ ++ ++ +/++
CD53 + + ++ +d
CD81 ++ + + +
sIgH ++ + ++ +d
CD23 -/++ -/++ - -
CD25 - - + -
CD27 - - + ++
CD40 ++ ++ ++ +
CD86 - - - +
CD95 - - -/+ +
CD200 N.A. + - N.A.
HLA-DR ++ ++ ++ +/++
CXCR4 -/+d + + -/+d
CXCR5 + + + -/+d
CCR6 + + + -
CD138 - - - -/+d
TLR9 N.A. + ++ ++
CD10 + - - -
CD24 + +d + -
CD38 + -/+d -/+d +++
CD43 - - - +
CD53 + + ++ +d

FIG. 3. Overall immunophenotypic profiles of different populations of circulating PB B-cells in healthy adults. N.A.: not analyzed.

linked lymphoproliferative disease), including BM regeneration after transplantation (16,17). This phenomenon is associated with decreased numbers of memory B-cells (12–14). The possibility that in humans some of these cells can conclude their maturation in the periphery, act as human regulatory B-cells (75), or undergo neoplastic transformation, remains to be investigated.

IMMUNOPHENOTYPIC CHARACTERISTICS OF HUMAN PB B-CELL SUBSETS AND THEIR FUNCTIONAL CORRELATES

BCR-Associated Molecules

Ag-specific stimulation of B-cells is typically mediated by the BCR (sIg/CD79$_{a/b}$) coligation in the context of BCR coreceptor (CD19/CD21/CD81/CD225) and other surface molecule (CD20/CD45) costimulatory signals (76–78). The activation signal is counterbalanced by negative regulators such as CD5 and CD22, associated with signaling complexes that recruit phosphatases to protect from uncontrolled self-reactivity and even autoimmunity (78–81). The balance between BCR binding, the activation of the surface costimulatory molecules, and the negative regulators drives the first step of the Ag-induced response (76-78,82).

Consistent with their recent origin in the BM, both immature and naïve mature PB B-cells show homogeneous expression of most BCR-associated molecules (e.g.: CD19, CD20, CD22, and CD45—Fig. 3-); however, higher mean intensity of sIgM expression and slightly lower sIgD is detected in the immature vs. naïve mature

B-cells (12-15,74). In addition, in contrast to naïve mature B-lymphocytes, circulating immature B-cells also show a lower mean intensity of expression of the CD21 molecule (74), homogeneous positivity for CD5 and higher levels of CD81 expression (12–15). Although the CD81 tetraspanin adhesion molecule was initially described as being part of the BCR complex, now it is known to be associated with CD19/CD21/CD225 (83). Overexpression of CD5, together with decreased reactivity for CD21 in at least some of these cells, could prevent peripheral activation of immature B-cells, as suggested by their lower in vitro response to BCR stimulation (12–14). In addition to immature PB B-cells, CD5 is also expressed in a minor subset (15% ± 10%) of phenotypically naïve mature B-lymphocytes. However, no information is currently available about the functional capabilities of CD5- vs. CD5+ naïve mature B-cells.

Few differences have been observed in the expression of BCR-associated molecules between naïve and memory B-cells in PB. One important difference is the absence of CD5 expression on memory B-cells (15). Accordingly, the higher response of memory B-lymphocytes would not substantially rely on Ag-specific stimulation but on other signals such as those provided by T-cell costimulation. On the other hand, since plasmablasts are no longer dependent on Ag-stimulation, the BCR and all the BCR-associated molecules are downregulated in PB PC which show dim sIg (15% of PB PC are even sIg-), CD19 and CD45 expression, and absence of reactivity for CD5, CD20, and CD21, CD22; an exception is CD81 that remains positive on PB PC (15).

Although most circulating B-lymphocytes show high CD21 expression, there is a fraction of both naive and memory B-cells that express low CD21 levels (84,85). $CD21^{lo}$ B-cells (3%±2% of naïve and 4% ± 3% of memory B-cells) have phenotypic characteristics different from their $CD21^{hi}$ counterpart (84,85): expression of CD11c and CD95 in the absence of CD23 vs. a CD11c−, CD95−, CD23−/+ phenotype for $CD21^{hi}$ naïve/memory B-cells (Supporting Information Fig. 1). Although very little information has been reported about the biological role of $CD21^{lo}$ B-cells, their frequency has been found to be increased in both primary (e.g., common variable immunodeficiency) (85) and secondary (HIV infection) (84) immunodeficiencies.

Molecules Associated with T-Cell Dependent B-Cell Response

T-cells provide BCR-primed B-lymphocytes with costimulatory signals that are typically required by the latter cells to further proliferate and mature; such signals are delivered through both cell surface molecules and cytokines (2,3). After BCR binding to an antigen, the BCR-Ag complex is internalized and processed epitopes from the Ag are presented to CD4+ antigen-specific T-cells through HLA class II (2,3). Engagement of class II molecules allows interaction of CD40 on B-cells with its T-cell ligand CD154, inducing: (i) transcriptional factors required for the development of germinal centers, (ii) changes in the expression of surface molecules, (iii) proliferation, (iv) somatic hypermutation, (v) IgH class-switching, and (vi) differentiation to memory/effector compartments (3). Although CD40 is not essential for the generation of IgM-secreting cells (3), it plays an essential role in B-cell activation and CSR (3,86). Response to T-helper (Th) cytokines is also important for B-cell activation, and both in vitro CD40 ligation and IL-2 signaling lead to B-cell proliferation and differentiation (87). Immature, naïve mature, and memory B-cells show a homogeneously high levels of expression of CD40 and HLA-DR (12,15), while expression of the IL-2 receptor α-chain (CD25) is only detected on a subset of memory B-lymphocytes (15,88), which respond in vitro to IL-2 stimulation (57,59). In addition, most memory B-cells are positive for CD27 (9,10), a molecule that is upregulated during the latest stages of the GC reaction (3). In contrast, CD23 expression is expressed on a fraction of all immature B-cells (68% ± 17%) and naïve mature B-cells, but absent in the memory compartment. Since CD23 strengthens the proliferation and survival during initial phases of the GC reaction (89), the different patterns of expression of CD23 in naïve vs. memory B-lymphocytes suggests that reactivation of memory B-cells starts in a later step than that of naïve B-lymphocytes, which are now independent of CD23 signaling. In turn, CD200 is expressed on normal PB naïve B-cells but downregulated in the memory compartment. Although there is no definitive information about the role of CD200 in B-cells, it has been shown that expression of this marker by chronic lymphocyte leukemia B-cells suppresses T-cell associated cytokine secretion, including IL-2, through its engagement with CD200R (90). Accordingly, this molecule could act as a negative regulator of naïve B-cell activation, downregulating paracrine cytokine secretion by T-cells; in such case, memory B-cells would be independent from this specific control.

A change in the profile of expression of T-cell associated B-cell molecules is observed in PB PC vs. both naïve and memory B-cells (15). Thus, those molecules required for the interaction of B-cells with lymph node T-cells are downregulated (CD25/CD40) or heterogeneously expressed (HLA-DR), and they are replaced by other T-cell dependent costimulatory proteins such as CD27 and CD86 (15,62). Signalling through these new co-stimulatory molecules is now associated with Ab-secretion (91), suggesting that the pattern of interaction with T-cells is different for Ag-specific response and Ab-secretion support.

Although CD95 (Fas) is upregulated during the GC reaction (91), it is negative in almost all PB memory B-cells. In contrast, most circulating PB PC are CD95+ (15), probably reflecting a preprogramming to death for the short life effector cells. Accordingly, in contrast to memory B-cells that are long-lasting, most newborn plasmablasts die soon after they have been generated, except for those which are able to compete with settled PC for a survival niche e.g. in the BM (37). Plasmablasts which are not able to settle would be deleted or

destroyed by T-cells or other CD95L expressing cells (e.g., NK cells) (92).

Molecules Driving B-Cell Traffic

Although there is much promiscuity within the chemokine network, and a single molecule can signal for different tissues, certain combinations of chemokines and chemokine receptors guide B-cells to specific tissues (93). Accordingly, CXCR4, CXCR5, CCR6, and CCR7 have been identified as receptors that drive B-cells to LN attracted by a combination of CXCL12 (SDF-1), CXCL13, CCL20 and CCL19, respectively (93). CXCR4 can also drive B-cells to home in the BM in response to CXCL12 production by BM stromal cells (37,93), whereas CCR9 and CCR10 have been associated with homing to peripheral tissues (e.g. MALT) through the CCL25 and CCL28 chemokines (61,93), and CXCR3 would lead B-cells to sites of inflammation (93).

Expression of CXCR4, CXCR5, CCR6, and CCR7 has been reported on circulating naïve and memory B-cells (15,94). CCR6 has also been detected on immature PB B-cells (15), but the intensity of CXCR4 and CXCR5 is lower on these latter cells (13). In contrast, dim-to-negative expression of CXCR4, CXCR5, and CCR6 is detected on PB PC (15,61,63,67). According to their suggested mucosal origin, some groups have reported increased CCR9 and CCR10 on the circulating plasmablasts (61,62,67), although this could not be confirmed by others (15).

Toll-Like Receptors

Toll-like receptors (TLR) are a group of glycoproteins that recognize a diverse array of ligands sharing molecular motifs conserved within different pathogens. Signaling through these receptors is non-specific, but it enhances activation of lymphocytes and other immune cells contributing to Ag-specific responses (95). Surface expression of TLR1 and TLR6-10 has been reported on human B-cells (95), as well as dim surface and higher intracellular TLR9 expression (23). TLR9 is an intracellular protein present in the late endosome/lysosome that interacts with CpG DNA of processed antigens internalized after binding to the BCR, supporting activation of GC B-cells (95). Higher levels of TLR9 in memory/effector (CD27+) vs. naïve B-cells (23), together with the observation that TLR9 does not need BCR-triggering to promote CSR in memory B-cells (58), suggest that this receptor could have a major role during secondary immune responses.

Other B-Cell Molecules

CD10, CD24, and CD38 are three molecules associated with intracellular signaling and upregulated during B-cell development in the BM and the GC. CD10 and CD38 have enzymatic activity over the intracellular concentration of signaling peptides (CD10) (96) and Ca2+ (CD38) (97), and CD24 seems to be associated with repression of B-cell activating signals from non Ag-specific receptors (e.g., TLR) (98).

CD53 is a tetraspanin molecule that provides a positive signal triggering cell survival (99), although the ligand for this molecule and the stages of B-cell development in which it participates remain undetermined. Similarly, CD43 has been described as an activation marker restricted, among the B-cells, to PC and pro-B cell precursors (100), but very little information is available about its biological role.

According to their origin, immature PB B-cells still retain expression of markers upregulated during early phases of the B-cell development in the BM which are either not expressed (CD10/CD38), or show a lower intensity (CD24), in fully mature resting naïve and memory B-cells (12,15). However, since immature B-cells can leave the BM at different points during the later steps of maturation, they show heterogeneous expression of these markers ranging from higher levels in the most immature elements to those phenotypically and functionally closer to the naïve B-cell subset. In contrast, PB immature, naïve and memory B-lymphocytes share CD53 expression and lack CD43, the expression of the later marker being restricted to PB PC compartment which also shows the highest intensity of CD38 expression among all PB B-cell compartments (15).

Phenotypic Differences Between Plasmablasts and Plasma Cells

Although the $CD38^{hi}$ subset of PB B-cells can comprise both newly-generated plamasblasts and PC that recirculate from the BM, there are clear phenotypic differences between the circulating plasmablasts/PC and the PC that are attached to the BM. Those PC settled in the BM survival niches show lack of most of the pan-B markers (e.g., CD20 and CD22), heterogeneous expression of CD19, CD45, and $CD56^{lo}$, and high levels of CD38 and CD138 (101,102). In contrast, the PB fraction show dim-but-homogeneous expression of CD19 and CD45, absence of CD56, slightly lower CD38 expression than BM PC, and heterogeneous expression of CD20 and CD138 (15,61-62,103). Together with this, more than 75% of the circulating plasmablasts/PC show sIg^{lo} expression, while most of BM PC have completely downregulated BCR expression and are sIg^{-} (15).

More intriguing is the discrimination in the PB fraction between newly generated plasmablasts and those PC leaving the BM and recirculating. In steady state individuals, this fraction can be split in two subsets according to CD138 expression. Those PC with higher levels of CD138 show more mature phenotypic characteristics: higher expression of CD38 and cy-Ig, and lower CD45 levels (15). In contrast, no differences were reported between CD138− and CD138+ PB PC regarding expression of adhesion molecules and chemokine receptors (15). Interestingly, both compartments show high Ki-67 staining (15), suggesting that these cells are still completing maturation. In turn, newly generated plasmablasts after Ag-specific vaccination (Tetanus toxin) show high CD38 and CD138 positivity, and lack on CD20, as compared with steady state circulating PB plasmablasts/

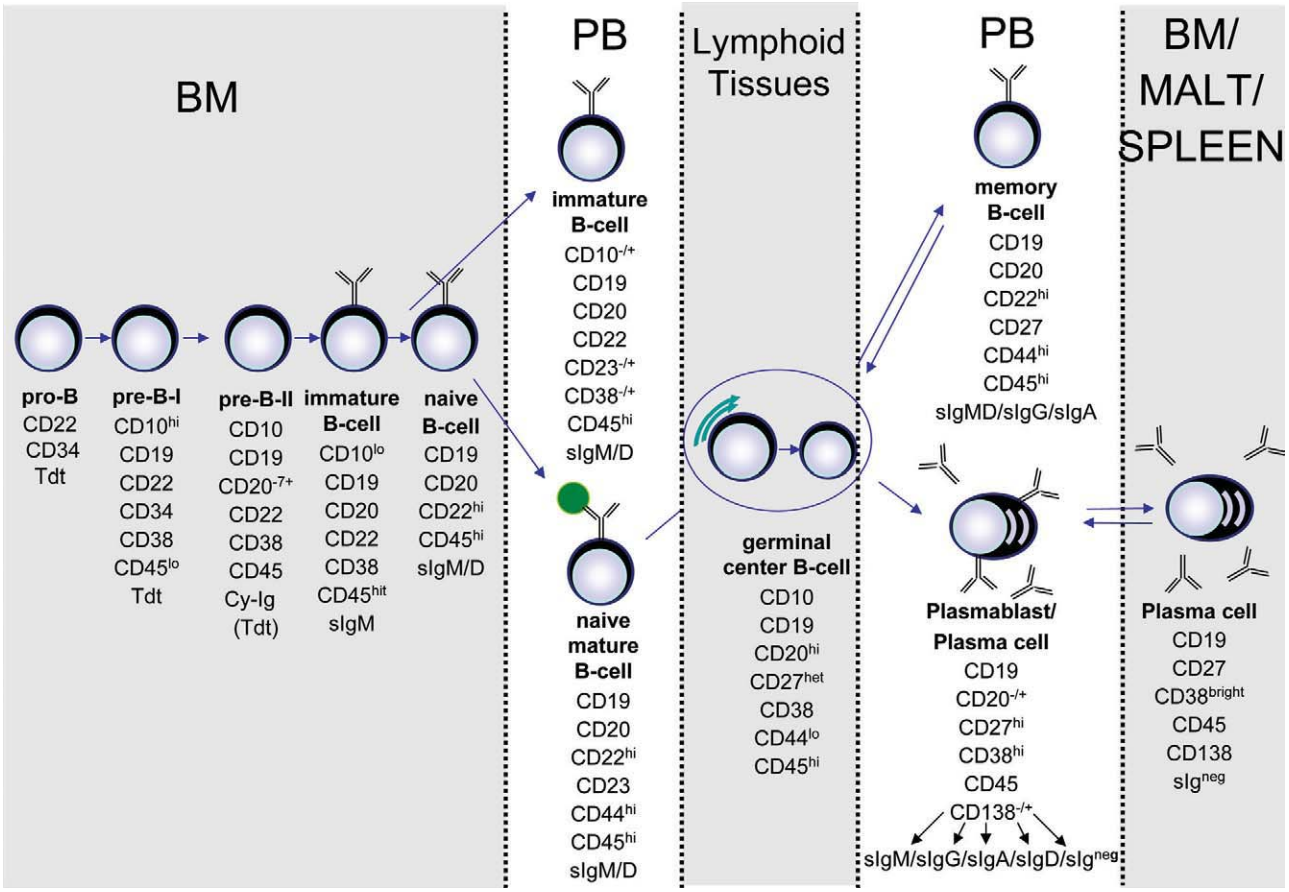

FIG. 4. Human B-cell differentiation subsets in the bone marrow (BM), peripheral blood (PB), secondary lymphoid tissues and mucosa-associated lymphoid tissues (MALT) classified according to their phenotypic characteristics and IgH isotype. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

PC (62,96). Accordingly, CD138 seems to be more probably related to post-GC maturation (15) or BM-driven migration (104), than a marker that discriminates between plasmablasts moving from secondary LN and PC leaving the BM.

AGE-ASSOCIATED CHANGES IN PB B-CELL COMPARTMENTS FROM HEALTHY SUBJECTS

During childhood, lymphocytes encounter many new antigens that stimulate their activation and proliferation until sufficient levels of specific immune surveillance and memory cells are reached and secondary lymphoid tissues are filled with mature B- and T-cells. Accordingly, the absolute number of PB B-cells shows a two-fold increase during the first 5 months of life from a median number of 600 B-cells/μL to 1,300 cells/μL; afterward, it decreases from age of 2 years to young adults (105). The number of circulating B-cells remains stable during adulthood and it only decreases in individuals older than 60 years (15). Although based on murine models, it has been suggested that this is due to a reduced BM ability to produce B-cells (106,107). No age-associated significant changes in the overall number of immature or naïve B-lymphocytes is observed in human adults (15). In contrast, significantly lower numbers of PB memory B-lymphocytes and PC have been found with increasing age particularly among individuals over 60 years (15,108). This suggests a reduced recruitment of naïve B-lymphocytes into memory B-lymphocytes and thereby, reduced PC in this age group. In principle, this should not be attributed to a lower ability for Ig CSR (108) since decreased numbers of both nonswitched IgM^+/IgD^+ and switched IgG^+ memory B-lymphocytes have been found with aging (15). Alternatively, this age-related decrease in memory B-cell counts among older adults could potentially be due to a lower exposure to new Ag (associated with a less-exposed lifestyle) leading to a more restricted memory B-cell repertoire or to an exhausted ability of memory B-lymphocytes that have been triggered many folds along the lifespan of elderly people, to generate expanded responses. This latter hypothesis could also lead to an increased rate of senescence, which could contribute to explain the high frequency of clonal premalignant B-cell conditions with genetic abnormalities such as CLL-like clones in monoclonal B-lymphocytosis (MBL) in elderly subjects (109,110).

The age-associated decrease in the number of circulating IgA^+, IgG^+, or IgM^+ PC is even more pronounced

than that of memory B-lymphocytes (15), suggesting the occurrence of diminished humoral response rates in the elderly. Current views suggest that circulating PC are mainly composed of new-born plasmablasts generated in the lymph nodes from Ag-triggered memory B-lymphocytes (61); once these cells have lost lymph node chemokine receptors, they are induced to circulate until finding an available niche in tissues such as the BM, spleen or MALT (93). Thus, the age-related decrease in the number of circulating PC may be a consequence of the decrease in memory B-lymphocytes, and these circulating PC would not be representative of the whole PC compartment in the body that may survive for many years. In line with this hypothesis, no significant correlation has been found between the number of IgH isotype-specific circulating PC and the serum concentration of their Ab counterpart (15). However, we should also consider whether a fraction of circulating PC could correspond to re-circulating BM and/or MALT- derived long living PC (7). In such a case, their decrease could be due to a decrease in these long-living PC because of limited PC niches, as described for elderly mice (111).

CONCLUDING REMARKS

Although PB is much more easily accessible than the BM or the LN tissues, lack of appropriate marker combination panels together with the phenotypic complexity of imbalanced subsets of circulating PB B-cells has hampered detailed accurate analysis and classification of PB B-subpopulations until $\geq$6-color flow cytometry became routinely available. According to their phenotypic profile (which also reflects their functional abilities and behavior) four major maturation-associated B-cell subsets can be identified in human PB: immature, naïve and memory B-lymphocytes, and plasma cells (Fig 4). Principal component analysis (15) has identified combined assessment of CD10, CD27, and CD38, as the best combination of markers for the discrimination among these B-cell populations in PB. Accordingly, the minimum panel required to get the whole profile of circulating B-cell subsets should include these three markers in addition to CD45 and a pan-B antigen. Since CD20 expression by PB plasmablasts is dim-to-negative, CD19 is recommended for the identification of all normal PB B-cells. The combination of CD19 with CD20 would allow a more accurate identification of the PB plasma cell compartment. Memory B-cells and plasmablasts can be further subclassified according to their sIg isotype: $sIgM^+$-only, $sIgM^+/sIgD^+$, $sIgG^+$ and $sIgA^+$, memory B-lymphocytes and $sIgM^+$-only, $sIgD^+$-only, $sIgG^+$, $sIgA^+$ and sIg^- plasma cells. Finally, further subsetting of these PB B-cell subsets is achieved through specific additional markers (e.g., CD138). CD138 expression by PC seems to be related to their degree of maturation, and can be potentially used to discriminate between newly generated $CD62L^+$ plasmablasts and $CD62L^-$ PC that egress the survival niches after being replaced; in turn, circulating immature B-cells show variable and heterogeneous expression of CD5, CD10, CD21, and CD23, among other markers.

ACKNOWLEDGMENTS

Perez-Andres performed experimental work, analyzed results, and designed and wrote the paper. Paiva, Nieto, Caraux, and Schmitz performed experiments and critically reviewed the manuscript. Almeida performed analysis of the results and critically reviewed the manuscript. Vogt, Marti, Rawstron, Van Zelm, Van Dongen, Johnsen, and Klein critically reviewed the data and wrote the manuscript. Orfao has designed the work, contributed to the interpretation of data, and wrote the manuscript.

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